

Control of Apoptosis Signaling by Apo2 Ligand

SCOT A. MARSTERS, ROBERT A. PITTI, JAMES P. SHERIDAN,
AND AVI ASHKENAZI

Department of Molecular Oncology, Genentech, Inc. 1 DNA Way,
South San Francisco, California 94080

ABSTRACT

Apo2 ligand (Apo2L, also called TRAIL) is a member of the tumor necrosis factor (TNF) cytokine family. The closest homolog of Apo2L is CD95 (Fas/Apo1) ligand, to which it has 24% amino acid sequence identity. Similar to CD95L, Apo2L activates rapid apoptosis in many types of cancer cells; however, whereas CD95L mRNA expression is restricted mainly to activated T cells, natural killer cells, and immune-privileged sites, Apo2L mRNA occurs in a wide variety of tissues. Most normal cells appear to be resistant to Apo2L's cytotoxic action, suggesting the existence of mechanisms that can protect against apoptosis induction by Apo2L. The first receptor described for Apo2L, called death receptor 4 (DR4), contains a cytoplasmic "death domain"; DR4 transmits the apoptosis signal carried by Apo2L. We have identified three additional receptors that bind to Apo2L. One receptor, called DR5, contains a cytoplasmic death domain and signals apoptosis much like DR4. The DR4 and DR5 mRNAs are expressed in many normal tissues and tumor cell lines. The second receptor, designated decoy receptor 1 (DcR1), is a phospholipid-anchored cell-surface protein that lacks a cytoplasmic tail. The third receptor, called DcR2, is structurally similar to DR4 and DR5 but has a truncated cytoplasmic death domain and does not transmit a death signal. The mRNAs for DcR1 and DcR2 are expressed in multiple normal tissues but in few tumor cell lines. Transfection experiments indicate that DcR1 and DcR2 act as decoys that prevent Apo2L from inducing apoptosis through DR4 and DR5. These decoy receptors thus represent a novel mechanism for regulating sensitivity to a pro-apoptotic cytokine directly at the cell's surface. The preferential expression of these inhibitory receptors in normal tissues suggests that Apo2L may be useful as an anticancer agent that induces apoptosis in cancer cells while sparing normal cells.

I. Introduction

Apoptosis is a cell-suicide program that enables metazoans to eliminate individual cells that are no longer needed (Steller, 1995; Jacobson *et al.*, 1997). Apoptosis plays a central role in the control of tissue cell numbers during development and homeostasis, together with essential functions such as cell proliferation and differentiation (Steller, 1995). In the developing embryo, cells die by apoptosis during morphogenesis or synaptogenesis. In the adult animal, cells undergo apoptosis during tissue turnover or at the end of an immune response. Because the physiological role of apoptosis is critical, inappropriate initiation of this process can be pathological. Thus, aberrant apoptosis of certain brain neurons

Control of Apoptosis Signaling by Apo2 Ligand

SCOT A. MARSTERS, ROBERT A. PITTI, JAMES P. SHERIDAN,
AND AVI ASHKENAZI

*Department of Molecular Oncology, Genentech, Inc. 1 DNA Way,
South San Francisco, California 94080*

ABSTRACT

Apo2 ligand (Apo2L, also called TRAIL) is a member of the tumor necrosis factor (TNF) cytokine family. The closest homolog of Apo2L is CD95 (Fas/Apo1) ligand, to which it has 24% amino acid sequence identity. Similar to CD95L, Apo2L activates rapid apoptosis in many types of cancer cells; however, whereas CD95L mRNA expression is restricted mainly to activated T cells, natural killer cells, and immune-privileged sites, Apo2L mRNA occurs in a wide variety of tissues. Most normal cells appear to be resistant to Apo2L's cytotoxic action, suggesting the existence of mechanisms that can protect against apoptosis induction by Apo2L. The first receptor described for Apo2L, called death receptor 4 (DR4), contains a cytoplasmic "death domain"; DR4 transmits the apoptosis signal carried by Apo2L. We have identified three additional receptors that bind to Apo2L. One receptor, called DR5, contains a cytoplasmic death domain and signals apoptosis much like DR4. The DR4 and DR5 mRNAs are expressed in many normal tissues and tumor cell lines. The second receptor, designated decoy receptor 1 (DcR1), is a phospholipid-anchored cell-surface protein that lacks a cytoplasmic tail. The third receptor, called DcR2, is structurally similar to DR4 and DR5 but has a truncated cytoplasmic death domain and does not transmit a death signal. The mRNAs for DcR1 and DcR2 are expressed in multiple normal tissues but in few tumor cell lines. Transfection experiments indicate that DcR1 and DcR2 act as decoys that prevent Apo2L from inducing apoptosis through DR4 and DR5. These decoy receptors thus represent a novel mechanism for regulating sensitivity to a pro-apoptotic cytokine directly at the cell's surface. The preferential expression of these inhibitory receptors in normal tissues suggests that Apo2L may be useful as an anticancer agent that induces apoptosis in cancer cells while sparing normal cells.

I. Introduction

Apoptosis is a cell-suicide program that enables metazoans to eliminate individual cells that are no longer needed (Steller, 1995; Jacobson *et al.*, 1997). Apoptosis plays a central role in the control of tissue cell numbers during development and homeostasis, together with essential functions such as cell proliferation and differentiation (Steller, 1995). In the developing embryo, cells die by apoptosis during morphogenesis or synaptogenesis. In the adult animal, cells undergo apoptosis during tissue turnover or at the end of an immune response. Because the physiological role of apoptosis is critical, inappropriate initiation of this process can be pathological. Thus, aberrant apoptosis of certain brain neurons

contributes to disorders such as Alzheimer's and Parkinson's diseases, whereas failure of dividing cells to initiate apoptosis after sustaining severe DNA damage contributes to uncontrolled proliferation and cancer (Thompson, 1995).

Cells sometimes die accidentally, from physical or chemical trauma. In accidental cell death (called necrosis), the damaged cells swell, lyse, and release noxious contents into the extracellular space. This usually triggers an inflammatory reaction that can become harmful to the organism. Physiologic apoptosis, in contrast, is an orchestrated process in which the dying cells dismantle and package themselves for engulfment by neighboring cells. During apoptosis, cells execute numerous biochemical reactions: they reorganize their actin cytoskeleton, they cleave some of their own proteins, and they fragment their chromosomal DNA. At the same time, the cells display dramatic morphological changes. At first, they project and retract balloon-shaped plasma membrane extensions (membrane blebbing). Then, they condense their nucleus and cytoplasm and assemble into membrane-contained apoptotic bodies, marked for phagocytosis by other cells. Thus, apoptosis has evolved as a means of removing unwanted cells "quietly," without further consequence to the animal.

Metazoan cells are inherently programmed to die and will execute apoptosis if they don't receive appropriate survival signals from their environment (Jacobson *et al.*, 1997). This is important for preventing cells from growing outside of their appropriate location and for eliminating cells that carry out a function that is no longer relevant to the animal's survival. For example, neurons will automatically initiate apoptosis if they are deprived of neurotrophic factors or if they do not receive electrical stimulation. Besides an ability to sense external survival signals, metazoan cells have evolved internal sensors for well being. When a cell detects internal damage, it usually tries to repair it; however, if repair is not possible, the cell initiates apoptosis. Similarly, if a cell receives simultaneous but conflicting signals that drive it to proliferate or to arrest its division cycle, the cell triggers apoptosis (Steller, 1995; Jacobson *et al.*, 1997).

In addition to survival signals and to surveillance for intracellular integrity, higher metazoans have developed instructive signaling mechanisms that can direct cells to execute apoptosis. For instance, it is important for the animal to prevent excessive accumulation of activated lymphocytes in peripheral tissues, because these cells produce high levels of cytokines that can be harmful to the organism. Mechanisms that instruct activated T cells eventually to die by apoptosis (peripheral deletion) help to maintain the homeostasis of lymphocytes in the periphery (Nagata, 1997).

The initiation of apoptosis in response to survival factor withdrawal or intracellular malfunction is a multistep signaling process that usually requires new protein synthesis. In contrast, the initiation of instructive apoptosis is direct and it is typically augmented by inhibitors of protein synthesis. Cell-surface receptors,

called "death receptors," detect signals carried by specific "death ligands" and rapidly ignite the cell's apoptotic caspase machinery, leading to the cell's demise.

II. Death Receptors

The death receptors known to date belong to the tumor necrosis factor (TNF) receptor gene superfamily, which is defined by the presence of related, cysteine-rich, extracellular domains. The best-characterized death receptors are CD95 (also called Fas or Apo1) and the type I receptor for TNF (TNFR1, also called p55 TNFR) (Nagata, 1997; Ashkenazi and Dixit, 1998). A third death receptor, called DR3 or Apo3, was identified recently by a number of groups (for a review, see Ashkenazi and Dixit, 1998). The death receptors form a subgroup within the TNFR superfamily of receptors that contains a homologous cytoplasmic sequence termed the "death domain" (Tartaglia *et al.*, 1993). Death domains typically enable death receptors to engage the cell's apoptotic machinery but, in some cases, they also mediate functions that are distinct from apoptosis, such as activation of the transcription factor nuclear factor- κ B (NF- κ B). Some adaptor molecules that transmit signals from death receptors also contain death domains.

CD95, TNFR1, and DR3 signal apoptosis through the death domain-containing adaptor FADD/Mort1 (Chinnaiyan *et al.*, 1995; Boldin *et al.*, 1995). Fas binds to FADD directly; TNFR1 and DR3 bind FADD indirectly, through another adaptor called TRADD (Hsu *et al.*, 1995). FADD also contains a "death effector domain" that binds to an analogous domain repeated in tandem within the zymogen form of caspase 8 (also called FLICE, or MACH) (Boldin *et al.*, 1996; Muzio *et al.*, 1996). Upon recruitment by FADD, caspase 8 oligomerization drives its activation through self cleavage (Muzio *et al.*, 1998). Caspase 8 then activates downstream effector caspases such as caspase 9—the mammalian functional homolog of the cell death-controlling *C. elegans* gene CED-3—committing the cell to apoptosis. Studies with FADD gene knockout mice (Yeh *et al.*, 1998; Zhang *et al.*, 1998) establish that FADD is essential for apoptosis induction by CD95, TNFR1, and DR3.

III. Apo2 Ligand

The ligands for CD95 and TNFR1 are structurally related and belong to the TNF cytokine family (Gruss and Dower, 1995). We have recently identified a ligand for DR3/Apo3, termed Apo3 ligand (Marsters *et al.*, 1998). In earlier work, we identified a different molecule, termed Apo2 ligand (Apo2L) (Pitti *et al.*, 1996). We discovered Apo2L by searching expressed sequence tag (EST) databases for overlapping ESTs that showed sequence homology to members of the TNF cytokine family. On the basis of one such set of ESTs, we isolated a full-length cDNA that encoded a previously unknown TNF family member. We named this molecule Apo2L, because it showed the most sequence homology to

the ligand of CD95/Fas/Apo1 (Pitti *et al.*, 1996). The same molecule was identified independently by another group (Wiley *et al.*, 1995), who named it TRAIL. Apo2L is a 281 amino acid, type 2 transmembrane protein. The C-terminal, extracellular region of the protein is cleaved from the cell surface by proteases and forms a soluble, homotrimeric molecule.

Apo2L activates apoptosis in many tumor cell lines, as evidenced by hallmarks such as DNA fragmentation, morphological changes, or exposure of phosphatidylserine on the cell surface. Many tumor cell lines start membrane blebbing within minutes of exposure to Apo2L and complete the apoptotic death program within 1 to 6 hours.

CrmA, a poxvirus-derived serpin, blocks cell death induced by Apo2L, indicating caspase involvement in this response. In contrast, a dominant-negative mutant of FADD, which blocks apoptosis-induction by CD95, TNFR1, or DR3, does not block Apo2L function (Marsters *et al.*, 1996), suggesting that a different adaptor may be involved in death signaling by Apo2L.

IV. Death Receptor 4

In 1997, Vishva Dixit's lab reported cloning a receptor for Apo2L, which was named death receptor 4 (DR4) (Pan *et al.*, 1997). DR4 is a previously unknown member of the TNFR gene superfamily; it has two extracellular, cysteine-rich domains and a cytoplasmic death domain. Upon overexpression, DR4 activates apoptosis, which is blocked by CrmA but not by dominant-negative FADD (Pan *et al.*, 1997), suggesting that DR4 signals apoptosis through a FADD-independent, caspase-dependent pathway. These data are consistent with our observations for Apo2L itself (Marsters *et al.*, 1996).

V. Death Receptor 5

We independently cloned a different receptor for Apo2L, which we named initially Apo2, and later, DR5 (Sheridan *et al.*, 1997). DR5 was identified also by a number of other groups (for a review, see Ashkenazi and Dixit, 1998). DR5 is related most closely to DR4, to which it shows ~ 55% amino acid sequence identity. Like DR4, DR5 has two extracellular, cysteine-rich domains and a cytoplasmic death domain, which shows ~ 65% sequence identity to the death domain of DR4.

We tested whether DR5 binds to Apo2L by a co-immunoprecipitation assay. We labeled the receptor's extracellular domain (ECD) with a Flag epitope tag and the ligand with a polyhistidine tag. We were able to co-precipitate receptor-ligand complexes through the Flag epitope tag on the receptor and through the polyhistidine tag on the ligand. In addition, we fused the DR5 extracellular domain to the Fc region of an antibody, to generate a fusion protein that is sometimes referred to as an "immunoadhesin" (Chamow and Ashkenazi, 1996). This DR5

immunoadhesin blocked apoptosis induction by Apo2L, confirming specific interaction between DR5 and this cytokine (Sheridan *et al.*, 1997).

Upon overexpression in human HeLa cells or 293 cells, DR5 activated apoptosis, evidenced by morphological changes, DNA fragmentation, and phosphatidylserine exposure on the cell surface. DR5-induced apoptosis was blocked by CrmA or by peptide-based inhibitors of caspases. In contrast, DR5-induced apoptosis was not inhibited by dominant-negative FADD, suggesting that, like DR4, DR5 signals cell death through a FADD-independent pathway (Sheridan *et al.*, 1997).

We analyzed the mRNA expression of DR5 in human tissues and in a panel of eight cancer cell lines by Northern blot hybridization. We detected a single DR5 transcript in several fetal tissues and in adult tissues, including peripheral blood leukocytes (PBL), spleen, liver, and lung. In addition, we detected a relatively more abundant expression of the DR5 transcript in many tumor cell lines (Sheridan *et al.*, 1997). The broad expression of DR5 in normal tissues was surprising, because the Apo2L mRNA is also expressed in many tissues (Wiley *et al.*, 1995; Pitti *et al.*, 1996). This is in contrast to the mRNA expression of CD95L, which is restricted primarily to activated T cells and natural killer cells and to immune-privileged tissues such as the eye or the testis (Nagata, 1997). This observation suggests the potential existence of mechanisms that can protect normal cells from the cytotoxic action of this cytokine.

VI. Decoy Receptor 1

By using a signal sequence-trapping technique in yeast, we identified yet another new member of the TNF receptor family that showed close homology to protein DR4 and DR5. We subsequently named this decoy receptor 1 (DcR1). Like DR4 and DR5, DcR1 has two extracellular, cysteine-rich domains; however, the rest of the sequence is unique. There are five tandem pseudorepeats, each 15 amino acids long, followed by a stretch of hydrophobic residues, but there is no apparent cytoplasmic region. In fact, the C-terminal sequence of DcR1 is similar to sequences found in proteins that are tethered to the cell surface by a glycosylphosphatidylinositol (GPI) anchor. There are no charged residues downstream of the hydrophobic region; upstream of it are two amino acids with small side-chains, in this case, alanines (Sheridan *et al.*, 1997).

Since DcR1 seemed closely related to DR4 and DR5, we tested whether it binds to Apo2L. Flag epitope-tagged DcR1 bound to Apo2L in a co-immunoprecipitation assay. In addition, a DcR1-based immunoadhesin blocked apoptosis induction by Apo2L, whereas a TNFR1-based immunoadhesin did not. Also, cells transfected with DcR1 showed an increase in binding of radio-iodinated Apo2L, compared to cells transfected with empty vector. Further, treatment of DcR1-transfected cells with recombinant phosphatidylinositol phospholipase C (PI-

PLC), an enzyme that cleaves the GPI moiety, markedly reduced ligand binding, supporting the notion that DcR1 is a GPI-anchored receptor. These results indicate that DcR1 is a third receptor that binds to Apo2L (Sheridan *et al.*, 1997).

Because DcR1 lacks a cytoplasmic tail, we reasoned that it might be an inhibitory receptor that protects normal cells against killing by Apo2L. Transfection of human HeLa cells with an expression vector encoding DcR1 by itself did not trigger apoptosis; however, ectopic DcR1 expression substantially reduced sensitivity of the cells to induction of apoptosis by Apo2L. Treatment of 293 cells (which express the DcR1 mRNA) with PI-PLC sensitized the cells to killing by Apo2L but not by anti-Fas antibody. In addition, PI-PLC treatment of two types of primary endothelial cells—human umbilical vein endothelial cells (HUVEC) and human mammary microvascular endothelial cells (HUMEC)—that express high levels of DcR1 and are normally resistant to Apo2L caused marked sensitization to apoptosis induction by the ligand. These results support the notion that DcR1 is a GPI-linked decoy receptor for Apo2L (Sheridan *et al.*, 1997).

We analyzed the expression of DcR1 mRNA in tissues and in cancer cell lines by Northern blot hybridization. We detected several DcR1 transcripts in many normal tissues; the expression was most abundant in PBL. In contrast, we observed little or no expression of DcR1 transcripts in a panel of eight tumor cell lines, most of which are readily killed by Apo2L. These results suggest that DcR1 may serve a protective function against apoptosis induction by Apo2L, particularly in tissues that express the decoy receptor at high levels, by preventing the ligand from trimerizing its apoptosis-signaling receptors. In addition, the low abundance of DcR1 transcripts in tumor cells, which frequently express DR4 and/or DR5, suggests an explanation for why Apo2L can induce apoptosis in a variety of tumor cell lines. Thus, DcR1 represents a unique mechanism for regulating sensitivity to a death-inducing cytokine directly at the cell surface (Sheridan *et al.*, 1997).

VII. Decoy Receptor 2

To search for alternative forms of DcR1 that might contain a cytoplasmic region, we screened several cDNA libraries by hybridization to a DcR1-based probe; we found no such forms of DcR1. Instead, we identified a receptor that seemed to be a close but distinct relative of the three known Apo2L receptors. We subsequently named this protein decoy receptor 2 (DcR2) (Marsters *et al.*, 1997). DcR2 is organized topologically much like DR4 and DR5: it contains two extracellular, cysteine-rich domains and a cytoplasmic region with a sequence that resembles death domains. Surprisingly, the death domain of DcR2 is truncated: it is about one third the length of a typical death domain. Five out of six amino acid positions corresponding to residues that have been shown previously to be critical for apoptosis and NF- κ B signaling by TNFR1 (Tartaglia *et al.*, 1993)

and the position that corresponds to the site of the naturally occurring mouse *lpr* mutation in CD95 (Nagata, 1997) are absent in DcR2 (Marsters *et al.*, 1997).

We tested whether DcR2 binds to Apo2L. In a co-precipitation assay, the DcR2 ECD bound to the ligand; further, a DcR2-based immunoadhesin blocked Apo2L's ability to induce apoptosis. Thus, DcR2 is a fourth receptor that binds to Apo2L (Marsters *et al.*, 1997).

To test whether DcR2 triggers apoptosis, we overexpressed the receptor in HeLa or 293 cells. We observed no increase in cell death under conditions that allowed transfected DR4 or DR5 to trigger a robust apoptotic response. In addition, DcR2 transfection did not induce NF- κ B activation, whereas transfection of cells with DR4 or DR5 was associated with a weak NF- κ B activation. These results indicate that the truncated death domain of DcR2 is nonfunctional. We therefore reasoned that DcR2 might be an inhibitory receptor. Indeed, ectopic DcR2 expression substantially reduced cellular sensitivity to apoptosis induction by Apo2L. A DcR2 deletion mutant, lacking most of the receptor's cytoplasmic tail, was as effective as the full-length DcR2 at blocking Apo2L. These results indicate that DcR2 acts as a decoy, rather than transduces a signal that protects the cell against apoptosis induction by Apo2L (Marsters *et al.*, 1997).

We detected the DcR2 mRNA in many normal human tissues, with relatively high levels particularly in fetal liver and adult testis, suggesting that DcR2 might fulfill a protective function in these tissues (Marsters *et al.*, 1997). In contrast, we observed DcR2 expression only in a minority of tumor cell lines. Thus, like DcR1, DcR2 may fulfill a protective role in normal tissues against induction of apoptosis by constitutively expressed Apo2L. Because the topology of DcR2 is similar to that of DR4 and DR5, it is conceivable that this decoy receptor not only forms trimeric complexes with the homotrimeric ligand but also physically participates in mixed complexes that contain one or two death receptor molecules and, respectively, two or one decoy receptor molecules. Such mixed complexes are probably incapable of signaling, since, at minimum, trimerization of the death receptor is required for signal transmission.

It has been reported that a secreted TNFR homolog called osteoprotegerin (OPG), which is not closely related to the latter four receptors, also can bind to Apo2L and inhibit Apo2L's function (Emery *et al.*, 1998). OPG binds to another TNF family member that is involved in regulation of bone density (Lacey *et al.*, 1998); however, the interaction of OPG with Apo2L remains to be confirmed, as it was observed in the former study (Emery *et al.*, 1998) but not in the latter (Lacey *et al.*, 1998).

VIII. The Potential of Apo2L as an Anticancer Agent

The idea of targeting death receptors as a therapeutic approach to initiating apoptosis in tumor cells is intriguing; however, the clinical utility of targeting

CD95 or TNFR1 in cancer has been hampered by the severe toxic side effects that accompany the activation of these receptors. Injection of agonistic anti-CD95 antibodies in mice causes death within 1 day, due primarily to liver damage caused by apoptosis of hepatocytes, which express high levels of CD95 (Nagata, 1997). TNF injection initiates a lethal, systemic inflammatory response syndrome that resembles septic shock (Vassali, 1992). This inflammatory response is mediated by TNF activation of the transcription factor NF- κ B, which controls expression of many pro-inflammatory genes in vascular endothelial cells and in macrophages. In assessing the potential of Apo2L as a possible therapeutic agent, we compared its ability to activate NF- κ B with that of TNF. Apo2L induced weak activation of NF- κ B relative to TNF; furthermore, activation by Apo2L required three to four orders of magnitude higher concentrations of Apo2L than TNF. These data suggest that Apo2L is not a strong pro-inflammatory cytokine.

As a prelude for *in vivo* studies, we tested a panel of tumor cell lines for sensitivity to Apo2L-induced apoptosis. A wide variety of tumor cell lines from lymphoid as well as nonlymphoid origin showed sensitivity to Apo2L, including several cell lines derived from prevalent types of cancer (e.g., carcinomas of the lung, colon, breast, prostate). There was no linkage between the status of the p53 tumor-suppressor gene of these tumor cell lines and susceptibility to Apo2L, indicating that the ligand triggers apoptosis independently of p53. Because many cancer chemotherapeutic agents require functional p53 to trigger apoptosis in tumor cells, the finding that Apo2L induces apoptosis through a p53-independent signaling pathway suggested that combinations of chemotherapeutic drugs with Apo2L might achieve enhanced killing of tumor cells. We therefore tested a subset of tumor cell lines for sensitivity to established cancer chemotherapeutic agents in combination with Apo2L. We observed a substantially increased apoptosis of colon cancer cells exposed to 5-fluorouracil (5-FU) plus Apo2L and a similar effect on apoptosis of breast cancer cells treated with doxorubicin plus Apo2L.

To test whether Apo2L has antitumor activity *in vivo*, we used established models of subcutaneous human tumor cell xenografts in athymic mice. In a setting that examines the establishment of new tumors, treatment with daily injections of Apo2L, commenced 1 day after tumor cell injection, substantially reduced the frequency of tumor formation in the mice. In addition, in a model that examines the effect on growth of established tumors, Apo2L treatment caused significant tumor regression, while control tumors showed rapid growth. Additional studies in mice showed that Apo2L can cooperate with drugs such as 5-FU in preventing tumor growth. These results suggest that Apo2L might be useful as a single agent for treating tumors with mutant p53 that are refractory to chemotherapy or radiation treatment and also as an adjunct for conventional therapies for cancers that retain either partial or complete p53 function. Moreover, injection of Apo2L into

mice was not associated with any gross evidence of toxicity, suggesting that Apo2L may prove to be safe in other species and perhaps also in humans.

IX. Conclusion

Our results show that apoptosis signaling by Apo2L can be regulated by a family of receptors: two death receptors and two decoy receptors. DR4 and DR5 engage the caspase machinery through a FADD-independent signaling pathway. This was confirmed by a recent study with FADD gene knockout mice, which shows that FADD-deficient embryonic fibroblasts, which are resistant to apoptosis induction by Fas, TNFR1, and Apo3, are sensitive to apoptosis induction by DR4. The other two Apo2L receptors act as decoys that can provide protection against the cytotoxic action of the ligand. The genes encoding DR4, DR5, DcR1, and DcR2 map together to human chromosome 8p21-22, suggesting that they arose from a common ancestral gene. Whereas DR4 and DR5 are expressed in many normal tissues and cancer cell lines, DcR1 and DcR2 are expressed frequently in normal tissues but infrequently in tumor cells. As Apo2L mRNA is also widely expressed in normal tissues, it is likely that decoy receptor expression protects many cell types against Apo2L's cytotoxic activity, except perhaps under physiological circumstances in which receptor expression may be modulated specifically to increase cellular sensitivity to Apo2L. This differential pattern of death and decoy receptor expression also suggests that Apo2L may be useful as a therapeutic molecule that induces apoptosis in cancer cells while sparing normal cells. Initial studies in mouse models of cancer support this possibility. Future studies will explore the biological role of Apo2L as well as continue to assess the potential utility of this cytokine as a therapeutic agent for human cancer.

REFERENCES

- Ashkenazi, A., and Dixit, V.M. (1998). *Science* 280, 1305-1308.
- Boldin, M.P., et al. (1995). *J. Biol. Chem.* 270, 387-391.
- Boldin, M., Goncharov, T., Goltsev, Y., and Wallach, D. (1996). *Cell* 85, 803-815.
- Chamow, S.M., and Ashkenazi, A. (1996). *Trends Biotech.* 14, 52-60.
- Chinnaiyan, A.M., O'Rourke, N., Tewari, M., and Dixit, V.M. (1995). *Cell* 81, 505-512.
- Emery J.G., et al. (1998). *J. Biol. Chem.* 273, 14363-14366.
- Gruss, H.J., and Dower, S.K. (1995). *Blood* 85, 3378-3404.
- Hsu, H., Xiong, J., and Goeddel, D.V. (1995). *Cell* 81, 495-504.
- Jacobson, M.D., Weil, M., and Raff, M.C. (1997). *Cell* 88, 347-354.
- Lacey, D.L., et al. (1998). *Cell* 93, 165-176.
- Marsters, S.A., et al. (1996). *Curr. Biol.* 6, 750-752.
- Marsters, S.A., et al. (1997). *Curr. Biol.* 7, 1003-1006.
- Marsters, S.A., et al. (1998). *Curr. Biol.* 8, 525-528.
- Muzio, M., et al. (1996). *Cell* 85, 817-827.
- Muzio, M., Stockwell, B.R., Stennicke, H.R., Salvesen, G.S., and Dixit, V.M. (1998). *J. Biol. Chem.* 273, 2926-2930.
- Nagata, S. (1997). *Cell* 88, 355-365.

- Pan, G., *et al.* (1997). *Science* 276, 111-113.
 Pitti, R.M., *et al.* (1996). *J. Biol. Chem.* 271, 12687-12690.
 Sheridan, J.P., *et al.* (1997). *Science* 277, 818-821.
 Steller, H. (1995). *Science* 267, 1445-1449.
 Thompson, C.B. (1995). *Science* 267, 1456-1462.
 Tartaglia, L.A., Ayres, T.M., Wong, G.H.W., and Goeddel, D.V. (1993). *Cell* 74, 845-853.
 Vassoli, P. (1992). *Annu. Rev. Immunol.* 10, 411-452.
 Wiley, S.R., *et al.* (1995). *Immunity* 3, 673-682.
 Yeh, W.C., *et al.* (1998). *Science* 279, 1954-1958.
 Zhang, J., Cado, D., Chen, A., Kabra, N.H., and Winoto, A. (1998). *Nature* 392, 296-300.

DISCUSSION

Donald McDonnell: Have you had a chance to examine the combined effect of Apo2 ligand and anti-estrogens in mcf-7 cell xenograf models?

Avi Ashkenazi: We have not tested combinations of Apo2 ligand with anti-estrogens as yet. This is a useful suggestion and we plan to do such experiments. Incidentally, mcf-7 breast carcinoma cells are quite sensitive to apoptosis induction by Apo2 ligand as a single agent.

Aaron Hsueh: Are there viral genes similar to the decoy receptors?

Avi Ashkenazi: Poxviruses produce soluble homologs of the TNF receptor family that are believed to act as soluble decoys for certain TNF-related ligands. [For a review, see Smith *et al.* *Cell* 76, 959, 1994.]

Aaron Hsueh: Would you comment on the possible existence of splicing variants of death receptors that might act as decoy receptors?

Avi Ashkenazi: There is at least one such example: an alternatively spliced form of the Fas receptor, which is expressed by certain types of intrahepatic T cells [Hughes and Crispe. *J. Exp. Med.* 182, 1395, 1995].

Stephen Marx: This is a complex and growing group of protein families. It includes newly discovered molecules, like osteoprotegerin and TRANCE/RANKL, that cause growth and differentiation of osteoclasts. Can you give a broad outline of how this family causes both cell death and cell growth, depending upon the context?

Avi Ashkenazi: TNF receptor family members fall into two general categories: those receptors that lack cytoplasmic death domains and those that contain death domains. The family has 18 known mammalian members. Receptors without death domains generally signal through TRAF proteins, of which six are known so far. The effects include regulation of MAP kinase pathways, JNK/SAPK/p38 pathways, and NF-kappa-B; cellular responses include T cell activation, expression of immunomodulatory genes, and pro-inflammatory genes. Death domain-containing receptors signal mostly apoptosis but, in some cases, NF-kappa-B and JNK/SAPK/p38 pathways. These events are mediated by death domain adaptors such as FADD or TRADD and, in some cases, TRAF proteins. [For a current review of death receptors, see Ashkenazi, A., and Dixit, V. *Science* 280, 1305, 1998.]

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☒ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.